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Laser-induced damage in ocular tissue was studied with biochemical measures designed to characterize cellular damage mechanisms. Photochemical damage was identified by evidence of oxidative reactions resulting from photosensitizers and free radicals activated by the light exposure. Melanin, in the retinal pigment epithelial (RPE) cells, during illumination formed a free radical that rapidly oxidized ascorbic acid (AA). This specific reaction may safely direct excess photons into a chain of coupled redox reactions. RPE cells have a high capacity for utilizing AA; the cells have different transporters for AA and its oxidized form, dehydro-L-ascorbic acid (DHA), and are able to reduce DHA to AA. The kinetics of these transporters were measured in these studies. Light-activated melanin was also shown to react with linoleic acid, a model lipid. Thus, in the absence of sufficient AA, the melanin radical may initiate lipid peroxidation, a known concomitant of photochemical damage. Development of assays indicative of thermal damage was also started. Initial results suggested that extracellular potassium ion concentration increased following laser-induced thermal stress in RPE cells. This change was hypothesized to result from damage to sodium-potassium ionic pumps in the cell's plasma membrane.

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INVESTIGATION OF LASER-INDUCED RETINAL DAMAGE: WAVELENGTH AND PULSEWIDTH DEPENDENT MECHANISMS

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"Investigation of laser-induced retinal damage: wavelength and pulsewidth dependent mechanisms"

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Scope of Work

During the second year of this program, we have continued our research into the reactions initiated by light-activated melanin, in order to validate our hypothesis that melanin is serving as a photosensitizer which could be responsible for some types of photochemical damage. We have also begun to look at cellular changes relating to laser-induced, thermal damage, specifically changes in the cell's ability to regulate the ionic distribution across its plasma membrane. In this phase, we have utilized the newer and more sensitive technology of high performance capillary electrophoresis (HPCE) to measure ionic concentrations, as a supplement to the ion-selective electrodes which constituted the approach described in our original proposal. Although HPCE analysis is difficult to use for *in situ* measurements, (one advantage of the ion-selective electrodes), HPCE is ideally suited for measurements of aliquots taken from the cell extracts and cell cultures that we are beginning to utilize for studies on the effects of laser exposures on retinal pigment epithelium (RPE) cells. These techniques will be described in more detail below.

Further studies on the photooxidative reactions initiated by light-activated melanin.

The attached publication describing sodium-dependent ascorbic and dehydro-L-ascorbic acid uptake (Lam et al., 1993) is a result of our study of ascorbic acid utilization by RPE cells. The research demonstrated that the reduced and oxidized forms of ascorbic acid are taken up by the RPE cells by different transporters with different affinities. The ascorbic acid transporter has a high affinity but a relatively modest uptake velocity (V_{max}), while the dehydro-L-ascorbic acid (DHA) transporter has low affinity for its substrate, but a high V_{max}. Contrary to previous assertions by other workers, the presence in the RPE cell of high concentrations of DHA does not interfere or inhibit the uptake of ascorbic acid (AA). We have also demonstrated that once inside the RPE cell, DHA is quickly reduced to AA. Thus, one possible way to increase intracellular AA might be to introduce DHA. This is because, under normal physiological conditions, the concentration of AA in the plasma and ocular extracellular space is high enough to saturate the AA transporter, so that additional AA can not be immediately utilized by the cells. DHA is normally present at very low concentrations in the plasma, so that under these conditions the DHA transporter is idling. Exogenously applied DHA is quickly taken up by the cells and reduced to AA.

While this finding suggests obvious therapeutic applications, tempered by observations of possible toxicity of high doses of DHA, it also puts added emphasis on the importance of the

interaction of ascorbic acid with the light-activated melanin free radical, as well as the role of this system in photochemical light damage. The melanin-AA photooxidation reaction occurs quite rapidly, in a wavelength- and intensity-dependent manner. In our latest work (Glickman et al., 1993), we have measured the kinetics of this reaction. Under normal atmospheric conditions at room temperature, we measured a V_{max} for AA oxidation of 419 ± 70 pmols/min/10^6 RPE melanin granules. Interestingly, the V_{max} increases with increasing irradiation (Table 1), which indicates an increasing affinity of the melanin active site for AA, probably the result of additional free electron spins induced in the melanin heteropolymer by the increased laser irradiation. The high affinity for AA of the melanin active site suggests that this reaction is particularly well adapted to the task of safely channeling excess photon energy in the RPE into a chain of coupled, redox reactions, where the energy can be dissipated into chemical energy utilized by cytoplasmic systems. An undesirable alternative would be for the melanin radical to react with other cellular components, with possibly damaging consequences. We have studied the reaction of light-activated melanin with other cytoplasmic antioxidants, and have found little evidence of oxidation of glutathione (Fig. 1), tocopherol (vitamin E), and taurine (Lam & Glickman, 1992).

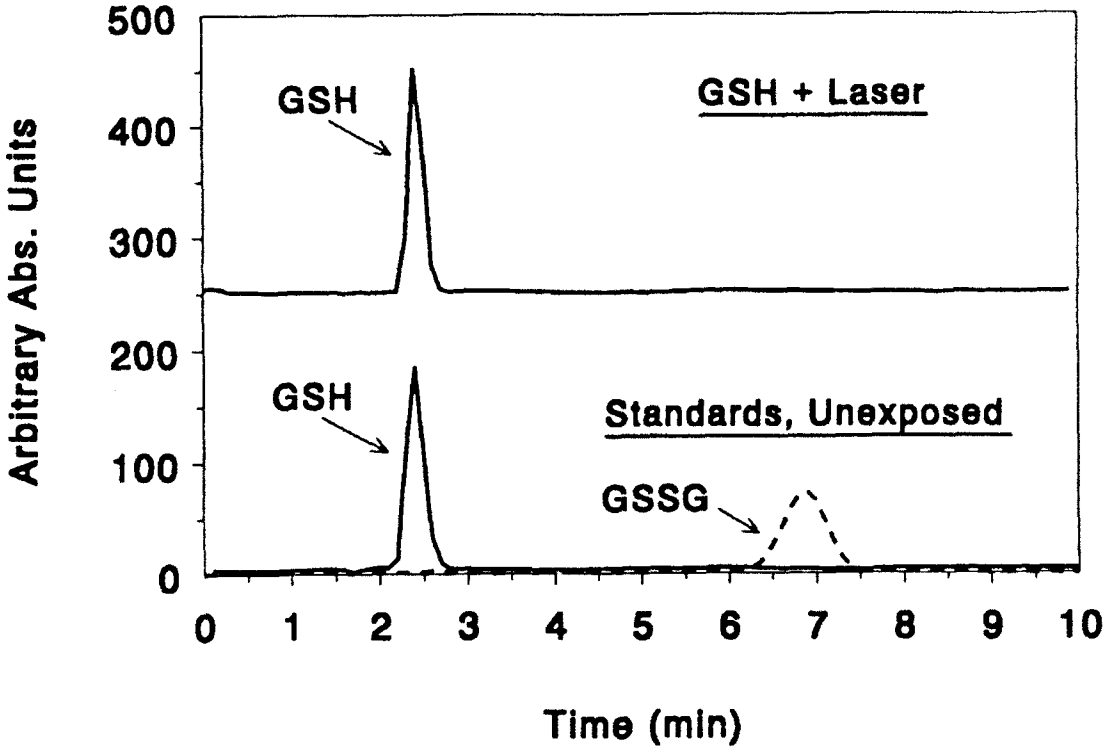

Fig. 1. Glutathione does not react with light-activated melanin granules. Top: Reduced glutathione (GSH) and melanin granules were exposed to laser for 2 min at irradiance of 400 mW/cm^2. A 2-µl aliquot was then injected into Delta HPLC column. No oxidized glutathione (GSSG) was detected. Bottom: GSH (200 nmol) and GSSG (32.6 nmol) standards separated on Delta column. [From Lam & Glickman, 1992.

Table 1. Effect of light intensity on K_m and V_{max} under standard or nitrogen atmospheres

	Standard Atmosphere		Nitrogen Atmosphere	
Laser (mW/cm^2) Avg ± Range	V_{max}[1] Mean ± S.D.	K_m (mM) Mean ± S.D.	V_{max}[1] Mean ± S.D.	K_m (mM) Mean ± S.D.
66 ± 1	419 ± 70	1.83 ± 0.59	not done	
170 ± 2	756 ± 94	1.32 ± 0.40	650 ± 285	1.01 ± 0.77
270 ± 3	871 ± 204	1.07 ± 0.62	769 ± 179	0.73 ± 0.33
369 ± 4	1118 ± 260	0.91 ± 0.52	not done	

[1] V_{max} expressed as pmols/min/10^6 melanin granules

Notes: Reaction mixtures containing labelled AA and approximately 5 x 10^6 melanin granules were exposed to the argon-ion laser at the indicated power densities in reaction vessels containing either air (columns under "Standard Atmosphere" heading) or nitrogen. All exposure durations were 1 min. AA oxidation was determined from the distribution of label in the AA and DHA fractions following separation on HPLC.

[from Glickman et al., 1993]

The selectivity of this reaction is rather surprising, considering data in the literature showing superoxide radical production during light irradiation (especially with UV wavelengths). In the biological milieu, superoxide quickly reacts with a multitude of biological molecules, or is transformed into H_2O_2 by the action of superoxide dismutase. Unless further broken down, e.g. by the action of catalase, hydrogen peroxide could generate reactive hydroxyl radicals. In any event, superoxide or hydroxyl radicals would be expected to oxidize many substrates. The lack of reactivity with other cellular antioxidants may be due to (1) inaccessibility of the melanin active site to other substrates because of steric hindrance, (2) a specific photooxidation reaction with the melanin radical directly without an oxygen radical intermediate, and/or (3) insufficient oxidation-reduction potential difference between the melanin radical and antioxidants other than AA. To test for the involvement of an oxygen radical intermediate, we measured the photooxidation kinetics under a nitrogen atmosphere. As shown in Table I above, the kinetics are little changed in anaerobic conditions. We also measured total DHA production under standard and nitrogen atmospheres (Table 2). Although the production of DHA was decreased under nitrogen, it was not abolished. Finally, H_2O_2 production during laser exposure was measured. Unexposed melanin contains endogenous H_2O_2, and the effect of the laser is to increase H_2O_2 slightly; the increase, however, is insufficient to account for the total oxidation of DHA (Table II). These results are reported in the manuscript to be published in *Free Radical Biology and Medicine* (Glickman et al., 1993). We hypothesize that our observations are consistent with the existence of two active sites on the melanin: the major one formed by an activated melanin, possibly an excited triplet state, and another, minor site formed through production of an oxygen radical, probably superoxide.

Table 2. DHA and hydrogen peroxide production during laser irradiation.

Non-Exposed		Laser-Exposed	
Std. Atm.	N_2 Atm.	Std. Atm.	N_2 Atm.
DHA (pmol)			
112 ± 40	131 ±32	493 ± 72	314 ± 56
H_2O_2 (pmol)			
714 ± 133	not done	956 ± 204	not done

Notes: All samples contained approximately 5 x 10^6 melanin granules. Ascorbic acid oxidation was determined by the production of DHA, detected with HPLC. The heading "standard atmosphere" designates that the reaction vessel was open to the laboratory air. The heading "nitrogen atmosphere" designates that the reagents and reaction vessel were purged with nitrogen prior to the experiment. Hydrogen peroxide production was measured under standard atmospheric conditions by HPLC. Samples were exposed to the mixed, blue-green output of the argon laser at 270 mW/cm^2 for one minute. The difference in mean H_2O_2 production between exposed and non-exposed samples did not reach statistical significance ($p \approx .09$, ANOVA followed by t-test).
[From Glickman et al., 1993]

Current Experiments

Lipid Peroxidation by Light-Activated Melanin

An important premise of our work is that light-activated melanin represents a reactive intracellular free radical which is capable of initiating photochemical damage to cellular components. Although we have found that the melanin radical is relatively unreactive to antioxidants other than ascorbic acid, the possibility exists that it could react with lipids, proteins, nucleic acids, or other cellular constituents. As a model of the lipid phase of the cell, we are using suspensions of linoleic acid, CH_3-$(CH_2)_3$-$(CH_2$-$CH{=}CH)_2$-$(CH_2)_7$-COOH, as a substrate for light-activated melanin. In these experiments, melanin granules ($\geq 10^6$ granules/ml) are extracted from RPE cells, washed in NaOH and then in saline buffer, and incubated with the linoleic acid (dispersed with lubrol). In the experiment illustrated in Fig 2, the mixture was exposed to the output of a Xe arc lamp at an irradiance of 0.4 W/cm^2 for various times as indicated. Aliquots are taken, filtered to remove melanin granules, placed on an μ-Bondapak-C18 HPLC column, and eluted with isocratic 65% acetonitrile-35% water solvent. Under these conditions, a light-dependent degradation of the linoleic acid is observed in the fraction eluting at 3.3 min. There is also a smaller fraction at 2.4 min showing changes related to the exposure. We think these represent formation of lipid peroxides, but this is a very recent observation and we have not yet characterized the reaction products. Nevertheless, this appears to be a very significant finding, and supports our hypothesis that the light-activated melanin radical can initiate photochemical damage in the cell. Obviously, we will be devoting significant time in the coming year to understanding this class of melanin-mediated reactions.

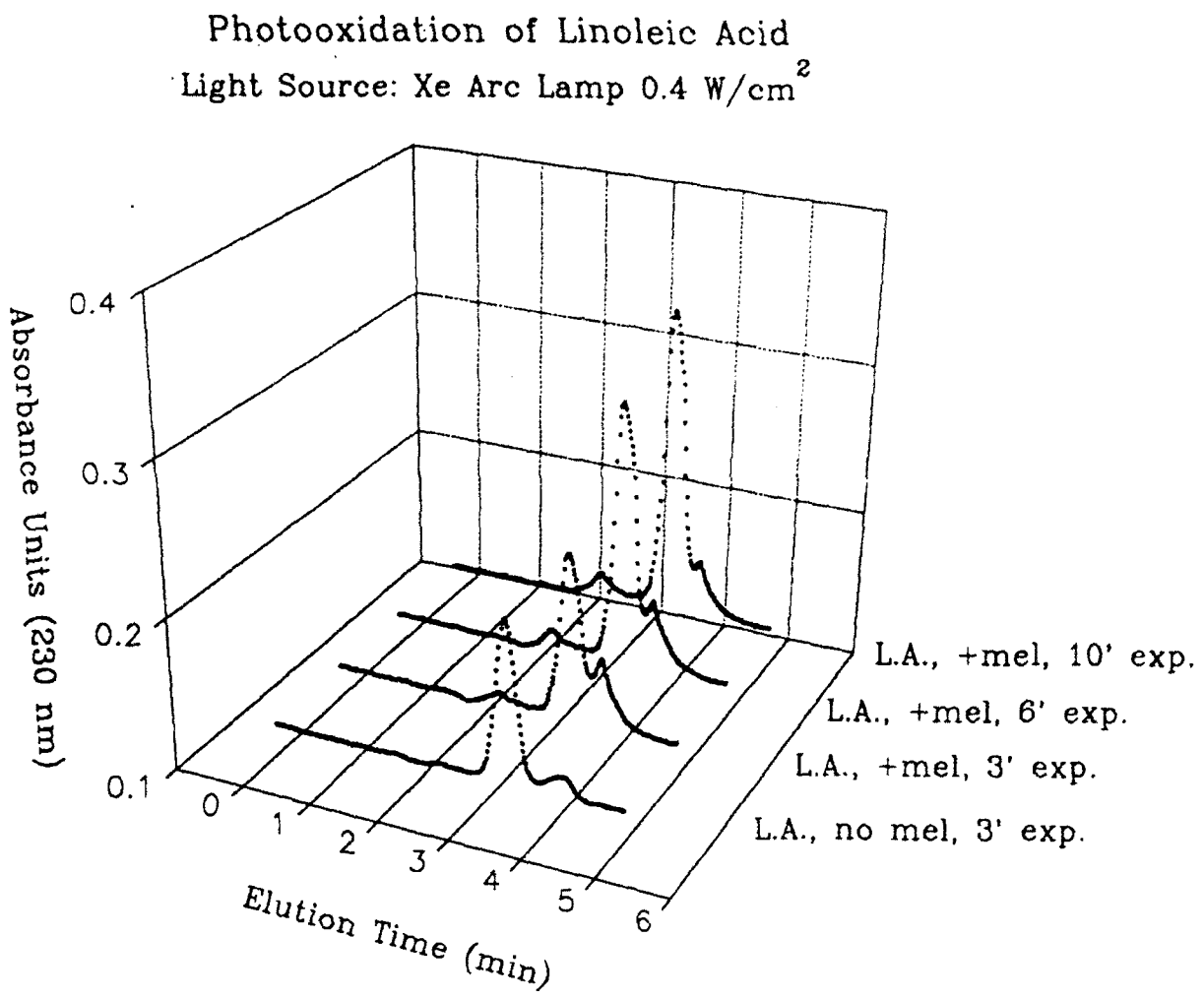

Fig. 2. Oxidative degradation of linoleic acid produced by light-activated melanin. Melanin granules (8.11 x 10^8/ml) were prepared from RPE cells obtained from fresh bovine eyes. The granules were added to a suspension of linoleic acid, and the mixture exposed to the full-spectrum output of a 150 W Xe arc lamp. At the end of the exposure, aliquots were assayed on HPLC as described in the text. Following increased light exposure, an increase in the amount of degradation products, probably lipid hydroperoxides was observed. In the absence of melanin, these changes were not found.

Effect of Thermal Damage on Ionic Balance

We have also started to examine the cellular reactions to other types of light damage. Following to the original proposal, we are measuring the changes in extracellular ionic balance, especially for sodium and potassium, following laser insults designed to produce thermal tissue damage. As mentioned in the Introduction, we felt that we needed greater sensitivity than offered by ion-selective electrodes to measure changes at exposure levels near the thermal damage threshold. The lower limit to the useful working range of the commercial electrodes we were using is about 0.1 mM for sodium, and about 0.05 mM for potassium. At these ion levels,

however, the signal is noisy and somewhat unreliable. In addition, the selectivity of the sodium electrode is only about 2 against potassium, and for the potassium electrode, the selectivity s about 4 against sodium (manufacturer's specifications). Therefore, at low concentrations, the potential error becomes rather significant. We were fortunate to be able to equip our laboratory this year with a high performance capillary electrophoresis machine which, among other applications, is able to detect cations and anions with good sensitivity and excellent selectivity. For example, a typical potassium calibration run is shown below (Fig. 3). The system gives a useful K^+ signal down to 10 μM or less, and the selectivity against Na^+ for dilute solutions is

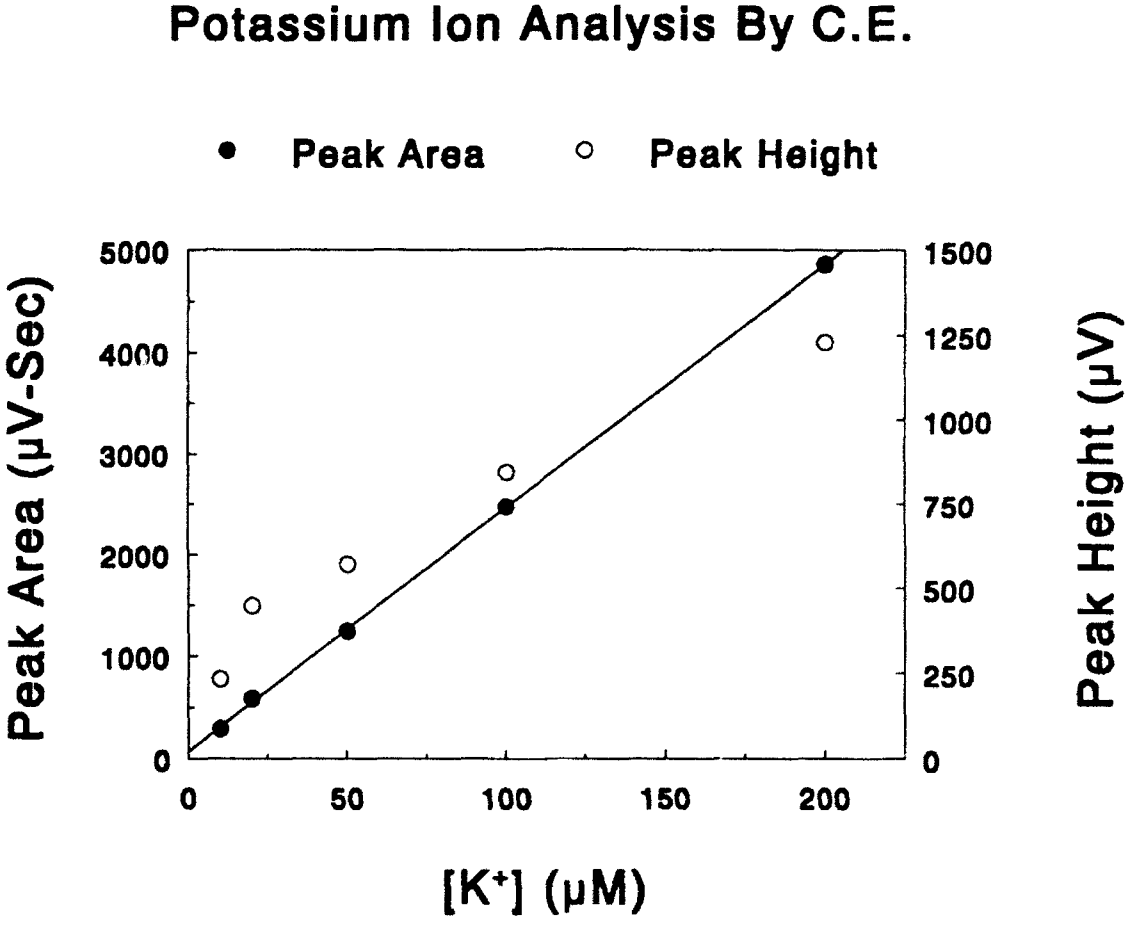

Fig. 3. Cation analysis performed on a Waters Quanta 4000 HPCE with 75 micron capillary. Ions were detected in cation HIBC buffer, with an electrophoretic potential of 20 kV. The results of K^+ analysis are shown in this figure. Peak area was a better metric than peak height for the electrophoretic analysis, based on linear proportionality with ion concentration.

close to 100%. (As the ion concentration increases, the peaks in the electrophoretogram widen to the point of overlap with adjacent ion peaks. At this point, it becomes difficult to determine the peak area with complete certainty. Fortunately, if this happens, one can simply dilute the sample

to put the ion concentrations into a useful range). The potential sensitivity of HPCE is quite impressive; recent technical improvements in the technique using laser-induced fluorescence may increase the detection sensitivity to as few as 100,000 ions, i.e. ~10^{-18} moles (reported in Science, vol 259, p 1260-1261, Feb 26 1993). It does not appear that we will need to increase our cation detection sensitivity to that level!

We have applied this analysis to isolated RPE cells, exposed to the blue green output of the argon ion laser. Viable cells (ascertained with trypan blue stain) were isolated from fresh bovine eyes and maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose. Suspensions of cells were placed in plastic microcentrifuge tubes and exposed to the laser. Following the exposure, cell-free aliquots were taken and subjected to cation analysis on HPCE. Results from one experiment are shown below (Table 3). The row marked "Medium" represents the DMEM alone, the row marked "Control" represents DMEM plus RPE cells before laser exposure, and the following rows are cells in DMEM exposed to the blue-green argon laser for 30 sec at the indicated power densities. The Na^+ and K^+ concentrations are shown, in mM, and the Na/K ratio calculated. Although we do not yet have enough data to draw a statistically valid conclusion, it appears that the extracellular K^+ concentration is starting to increase at the higher laser fluences. This change is in accordance with our original hypothesis that thermal damage to the plasma membrane would damage ATP-dependent ion pumps, resulting in a net leakage of K^+ from the cell. We note that Na^+ is also elevated, but not to the same proportion as K^+. We also note that after the 2.4 W/cm^2 exposure, the sample felt warm to the touch, so that this may have been the only exposure setting that actually produced thermal damage. It may be useful to have a continuous temperature monitor in contact with the sample to document the temperature change associated with the laser irradiation.

Table 3. Effect of laser exposure on extracellular Na^+ and K^+

Experimental Condition	Na^+ (mM)	K^+ (mM)	Na^+/K^+
Medium Only	159	4.2	38.0
Control (Unexp.)	138	4.9	28.2
1.1 W/cm^2	166	4.6	36.0
1.7 W/cm^2	164	4.9	33.6
2.4 W/cm^2	178	5.4	32.8

Notes: RPE cells were isolated from fresh bovine eyes and suspended in Dulbecco's modified Eagle's medium (DMEM). The cells were exposed to the blue-green (488 + 514.5 nm) output of an argon ion CW laser for 30 sec. Following the exposure, the cells were precipitated by centrifugation and an aliquot of the supernatant was analyzed for cations by HPCE. "Medium Only" was the DMEM alone; "Control" was a sham exposure of RPE cells in DMEM; the other rows were RPE cells in DMEM exposed to the laser at the specified power densities.

Future Plans

Clearly, this is only the beginning of the investigation into thermal damage mechanisms. Some problems which need to be addressed during the current phase include: (1) monitoring the temperature of the sample during exposure (difficult because a thermal-mechanical probe will absorb laser energy and itself be heated - possibly a remote, infrared temperature monitor could be used), and (2) obtaining a reliable source of viable RPE and retinal cells. Our current experiments indicate that the use of isolated, viable RPE cells is a productive approach to studying laser bioeffects on a cellular basis. We would like to culture the RPE cells in our laboratory to ensure a consistent supply, but we are not, at the moment, set up to do this. Our collaborator, Dr. Hing-Sing Yu, at the University of Texas at San Antonio (the sister Univ. of Texas campus in San Antonio), who cultured the transformed RPE cells used in our study of AA and DHA uptake (Lam et al., 1993), is on sabbatical this year and so that source of cells is not available to us. We expect Dr. Yu to return to San Antonio in the fall, and perhaps we will be able to re-establish a line of RPE and retinal cells then. In the interim, we will continue to use explants of cells from freshly obtained bovine eyes for the studies. The drawback to this approach is time and expense: the local slaughterhouse charges us $3.50 per eye, and it is usually late morning before the eyes can be brought back to the laboratory. Nevertheless, with careful planning, the experiments can be conducted with this approach.

Finally, in the upcoming year, we will start the cellular assays of nonlinear, photodisruptive damage. As we originally proposed, we will start by examining the release of cytoplasmic enzymes into the extracellular space. Lactate dehydrogenase and acid phosphatase are the two enzymes we selected as markers of membrane breakage. These enzymes are readily assayed using commercial kits. We do not foresee any technical difficulties in performing these experiments. We have a Q-switched Nd:glass laser installed in our laboratory which will provide 20-nsec pulses at 1060 and 530 nm. This will suffice for initial experiments. Other wavelengths, e.g. the 694 nm output of the ruby laser, will be obtained from from laser systems available at Brooks AFB.

Interactions

It is still planned to expose RPE and retinal cells to the 100-fsec output of the ultrashort pulse laser in the Laser Laboratory at Brooks AFB. We are continuing discussions with Drs. Roach and Rogers of AL/OEDL, concerning development of the best approach to studying the biological effects of the ultrashort pulses.

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